Bioorganic & Medicinal Chemistry Letters 19 (2009) 6544–6547

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A method for terminus proteomics: Selective isolation and labeling of N-terminal peptide from protein through transamination reaction

Kazuhiro Sonomura [a,b], Hiroki Kuyama [a], Ei-ichi Matsuo [a,b], Susumu Tsunasawa [a], Osamu Nishimura [a,*]

[a] Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Life Science Research Center, Technology Research Laboratory, Shimadzu Corporation, Kyoto 619-0237, Japan

ARTICLE INFO

Article history:
Received 9 September 2009
Revised 8 October 2009
Accepted 9 October 2009

Keywords:
Transamination
N-terminal labeling
MALDI-TOF MS
N-terminal sequence analysis

ABSTRACT

A novel method for selectively labeling and isolating N-terminal peptide from protein has been developed. An N^{α}-amino group of protein was converted to a carbonyl group through transamination reaction and the resulting carbonyl group was modified with *O*-(4-nitrobenzyl)hydroxylamine (NBHA). After proteolytic digestion using *Grifola frondosa* metalloendopeptidase (LysN), the modified N-terminal peptide remained unbound in the following treatment using amino-reactive *p*-phenylenediisothiocyanate (DITC) glass, whereas peptides other than the N-terminal peptide were effectively scavenged from the supernatant solution. The modified N-terminal peptide was thus successfully isolated and sequenced by matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-MS/MS) analysis.

Protein identification using mass spectrometry is a key technology in proteome research. In the conventional approach, such as peptide mass fingerprinting (PMF), target proteins are enzymatically or chemically digested into peptide fragments and measured mass values are compared with theoretical ones calculated from genome or protein databases.[1] Therefore, the exact state of mature proteins is not always determined; the mature protein sequence may often differ from that translated from its DNA sequence due to processes such as splicing and shuffling of mRNA, and/or the various post-translational modifications (PTMs). For conducting further study in depth on each protein identified by conventional proteome analysis, more detailed information about the exact amino acid sequence and PTMs of mature proteins from N-terminus to C-terminus has been highly desired.

Mass spectrometry has become a major tool in protein and peptide analysis and various methods have been developed and applied to actual samples.[2,3] However Edman sequencing is still the only conventional method for determining the N-terminal sequence. This method lacks generality, in that it cannot perform sequencing of N-terminally capped protein. Therefore, methodology using MS for both N- and C-terminal sequence analysis has been intensively studied for general and practical applications.[4–10]

We have been developing methods for isolating N- and C-terminal peptides from a peptide mixture of digested proteins and sequencing the isolated terminal peptides by MS.[8–10] In our review (in press), we proposed that this scientific field be termed 'terminus proteomics.'[11] In this study, we focused on the characteristic reactivity of metal-ion catalyzed transamination reaction,[12] which one of the authors (O.N.) had used for removing the N-terminal methionine residue from recombinant proteins produced from *Escherichia coli*.[13,14] This reaction selectively converts the N^{α}-amino group (N^{α}-NH_2) of peptides or proteins into a carbonyl group, whereas the coexisting N^{ε}-amino group (N^{ε}-NH_2) of lysine residue is kept intact. Reactions that selectively modify N^{α}-NH_2 or N^{ε}-NH_2 have often been utilized to isolate N- or C-terminal peptides from proteolytic digests; however, the selectivity is hardly obtained in general. The high selectivity of transamination reaction for N^{α}-NH_2 encouraged us to investigate its feasibility for isolating terminal peptides and we successfully developed a new method for C-terminal sequencing analysis using this transformation.[10] Here, we report an application of transamination reaction to N-terminal analysis, which was achieved by changing the enzyme used for digestion and by optimizing the procedure.

In the previous C-terminal analysis, proteins were first digested with lysylendopeptidase (LysC). LysC cleaves peptide bonds at the carboxyl side of lysine residues (-Lys-|-Xaa-) to yield mainly peptides having N^{α}-NH_2 and N^{ε}-NH_2 at both ends; however, C-terminal peptides have only N^{α}-NH_2. Subsequent transamination reaction converts the N^{α}-NH_2 to a carbonyl group; thus, incubation with amino-reactive *p*-phenylenediisothiocyanate (DITC) glass effectively scavenges N^{ε}-NH_2-containing peptides (peptides other than the C-terminal one). The recovered C-terminal peptide is open to modification with various nucleophilic reagents, such as hydrazine and hydroxylamine. We used 2,4-dinitrophenylhydrazine (DNPH), which demonstrated signal enhancement in MS analysis.

* Corresponding author. Tel./fax: +81 6 6879 4320.
E-mail address: osamu_nishimura@protein.osaka-u.ac.jp (O. Nishimura).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.10.044

In studying the C-terminal analysis employing a transamination reaction, we envisioned extending the method to N-terminal analysis by using *Grifola frondosa* metalloendopeptidase (LysN),[15–17] which cleaves proteins at the amino side of lysine residues, instead of LysC, as described in the previous report.[9] In addition, the protocol was changed so that a sample protein was first subjected to transamination and modification of the resulting carbonyl group, which facilitated the easy purification of the modified protein by simple gel filtration or ultrafiltration. Transamination reaction has been applied to proteins as well as peptides.[12,18–20] Thus, the procedure for N-terminal analysis was optimized (Scheme 1). The procedure consists of the following five steps: (1) transamination of proteins, (2) modification of the resulting carbonyl group through oxime formation, (3) LysN digestion, (4) treatment with DITC glass, and (5) MALDI -MS and MS/MS analysis. In this paper, sample proteins are transaminated and modified with *O*-(4-nitrobenzyl)hydroxylamine (NBHA) at their N-termini.[21] NBHA has a nitrobenzene moiety as well as DNPH, which would be expected to increase sensitivity in MALDI-MS analysis by combining a matrix system developed for sensitive detection of 2-nitrobenzenesulfenyl(NBS)-modified peptides.[22] LysN cleaves peptide bonds at the amino side of lysine residues (-Xaa-|-Lys-). After LysN digestion, the modified N-terminal peptide contains no amino group. Amino-reactive DITC glass scavenges the peptide fragments other than the N-terminal peptide, and the target peptide is left unreacted in the supernatant. Thus recovered N-terminal peptides are analyzed by MALDI-MS and MS/MS.

Two model proteins, bovine α-lactalbumin (α-la) and bovine β-lactoglobulin (β-lg), were chosen for testing the protocol. Proteins were transaminated in 10% pyridine containing 0.2 M glyoxylic acid, 6 mM $CuSO_4$, and 2 M urea. It was reported that the transamination reaction proceeded as well in the presence of urea.[12,14] Reaction time was set to 1 h, based on the results of our preliminary experiments using some peptides incorporating a different residue at their N-termini (data not shown). The reaction mixture was then subjected to a buffer exchange into 100 mM phosphate (pH 6.0) containing 2 M urea by ultrafiltration. Subsequent modification with NBHA was performed in 100 mM phosphate (pH 6.0) containing 50 mM NBHA-HCl and 2 M urea for 2 h at 37 °C. After a buffer exchange into 50 mM $NaHCO_3$ containing 2 M urea by ultrafiltration, disulfide bonds were reduced with tris(2-carboxyethyl)phosphine hydrochloride and the resulting free sulfhydryl groups were alkylated with iodoacetamide. LysN digestion was then performed at an enzyme-to-substrate ratio of 1:40 at room temperature for 15 h.[9] The upper panels in Figure 1 depict MALDI-MS spectra[23] after LysN digestion (Fig. 1a for α-la and Fig. 1c for β-lg), in which the modified N-terminal peptides (indicated by arrows) were detected among the peptide fragments as a sodium adduct ($[M+Na]^+$: 661.3 from α-la and $[M+Na]^+$: 976.5 from β-lg). When peptides containing a nitrobenzene moiety are analyzed by MALDI-MS, they are often accompanied by peaks with a 16 Da decrease probably due to splitting off of the oxygen atom from nitro group.[22] In both spectra these peaks were detected

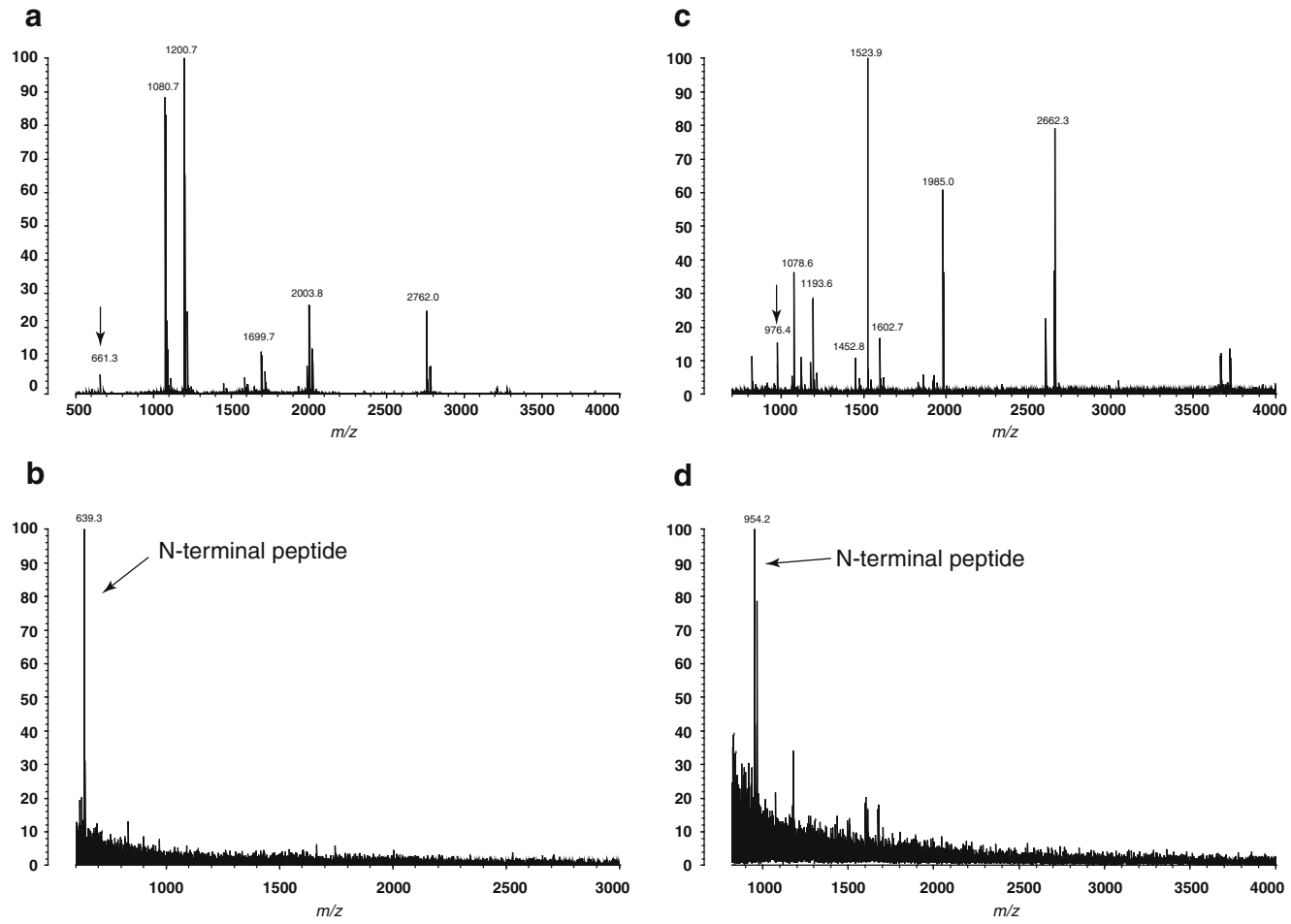


Figure 1. MALDI-MS spectra of peptide fragments of model proteins after LysN digestion ((a) α-lactalbumin, (c) β-lactoglobulin) and after treatment with DITC glass ((b) α-lactalbumin, (d) β-lactoglobulin.). Arrows indicate the modified N-terminal peptides of each protein. An aliquot (3 pmol of digest) was loaded onto the target plate.

and aided in discriminating the modified N-terminal peptides from the other peptide fragments even before the isolation step.

The LysN digest was then treated with DITC glass.[9,24] An aliquot of the digest (30 pmol) was added to pre-washed DITC glass. After 2 h incubation at 60 °C, the supernatant was directly analyzed by MALDI-MS. We employed a mixture of α-cyano-4-hydroxycinnamic acid (CHCA) and 3-hydroxy-4-nitrobenzoic acid (3H4NBA) as a binary matrix system, which was used for sensitive detection of NBS-modified peptides and was shown to be applicable to peptides containing a nitrobenzene moiety.[22,25] The lower panels in Figure 1 illustrate the MALDI-MS spectra after the DITC treatment (Fig. 1b for α-la and Fig. 1d for β-lg). Each N-terminal peptide was singly recovered ($[M+H]^+$: 639.3 from α-la and $[M+H]^+$: 954.5 from β-lg). In this experiment, these modified N-terminal peptides needed 3H4NBA as a matrix for the detection, the usefulness of which was also demonstrated in this case.

The isolated N-terminal peptides were subjected to MALDI-MS/MS analysis in the post-source decay (PSD) mode. The amino acid sequences of the peptides were analyzed using the observed fragment peaks (Fig. 2a for α-la and Fig. 2b for β-lg).

For N-terminally acetylated proteins, the method described here can be employed for N-terminal analysis because the N-terminal acetyl group is not affected in the transamination reaction (data not shown) and the acetylated N-terminal peptide after LysN digestion is free from the amino group. Studies in the case of N-blocked proteins were described in the previous reports.[5,6,9,26]

In the present study, we developed N-terminal analysis of proteins through transamination reaction. The method was applied to two model proteins, and successful isolation and sequencing of the N-terminal peptides were demonstrated. This method can not be used for some proteins (e.g., proteins containing N-terminal proline) because of limited reactivity for transamination.[10,27] However, an α-carbonyl of N-terminal ketoacyl group generated after transamination reaction can react with various types of nucleophilic reagents, and virtually any functionality can be introduced into the N-terminus of the peptide. This flexibility is advantageous for the MALDI-MS analysis of terminal peptides whose sensitivity largely depends on its amino acid composition, size, and modification states.

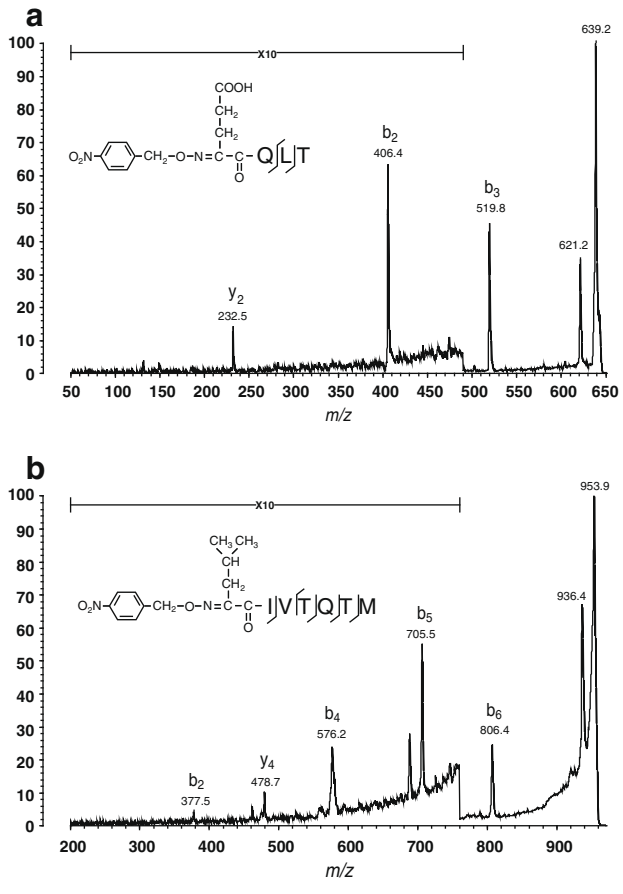
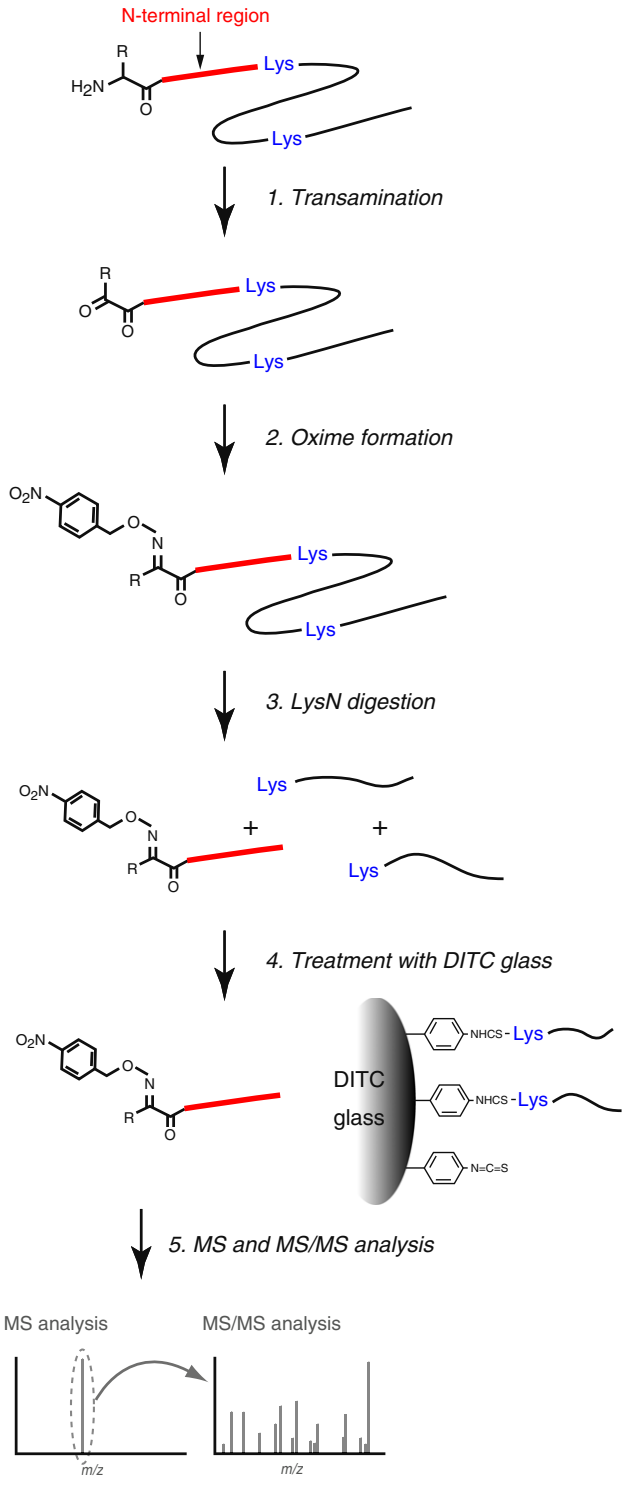


Figure 2. MALDI-MS/MS spectra (PSD mode) of the isolated N-terminal peptides of model proteins. (a) α-lactalbumin, (b) β-lactoglobulin.



Scheme 1. Protocol for selectively labeling and isolating N-terminal peptides.

References and notes

1. Henzel, W. J.; Billeci, T. M.; Stults, J. T.; Wong, S. C.; Grimley, C.; Watanabe, C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5011.
2. Mann, M.; Jensen, O. N. *Nat. Biotechnol.* **2003**, *21*, 255.
3. Thingholm, T. E.; Jensen, O. N.; Larsen, M. R. *Proteomics* **2009**, *9*, 1451.
4. Samyn, B.; Sergeant, K.; Castanheira, P.; Faro, C.; Beeumen, J. V. *Nat. Methods* **2005**, *2*, 193.
5. McDonald, L.; Robertson, D. H. L.; Hurst, J. L.; Beynon, R. J. *Nat. Methods* **2005**, *2*, 955.
6. Yamaguchi, M.; Nakayama, D.; Shima, K.; Kuyama, H.; Ando, E.; Okamura, T.; Ueyama, N.; Nakazawa, T.; Norioka, S.; Nishimura, O.; Tsunasawa, S. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 3313.
7. Mahrus, S.; Trinidad, J. C.; Barkan, D. T.; Sali, A.; Burlingame, A. L.; Wells, J. A. *Cell* **2008**, *134*, 866.
8. Kuyama, H.; Shima, K.; Sonomura, K.; Yamaguchi, M.; Ando, E.; Nishimura, O.; Tsunasawa, S. *Proteomics* **2008**, *8*, 1539.
9. Kuyama, H.; Sonomura, K.; Nishimura, O.; Tsunasawa, S. *Anal. Biochem.* **2008**, *380*, 291.
10. Sonomura, K.; Kuyama, H.; Matsuo, E.; Tsunasawa, S.; Nishimura, O. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 611.
11. We once used the term 'terminal proteomics' for the proteomics focusing on N- and/or C-terminal part of protein (Nakazawa et al. *Proteomics* **2008**, *8*, 673). However, we propose after careful consideration the term 'terminus proteomics' instead of former 'terminal proteomics'. The reason is that using 'terminus' removes any negative connotations and avoids misunderstanding or confusion. See the footnote in Kuyama, H.; Nishimura, O.; Tsunasawa, S. *Handbook on Mass Spectrometry: Instrumentation, Data and Analysis, and Applications*; Lang, J. K., Eds.; Nova Science Publishers: New York, in press; Chapter 19 (A New Method for N- and C-terminal de novo Amino Acid Sequencing of Proteins by Mass Spectrometry).
12. Dixon, H. B. F.; Fields, R. *Methods Enzymol.* **1972**, *25*, 409.
13. Nishimura, O.; Suenaga, M.; Ohmae, H.; Tsuji, S.; Fujino, M. *Chem. Commun.* **1998**, 1135.
14. Suenaga, M.; Ohmae, H.; Okutani, N.; Kurokawa, T.; Asano, T.; Yamada, T.; Nishimura, O.; Fujino, M. *J. Chem. Soc., Perkin Trans. 1* **1999**, 3727.
15. Nonaka, T.; Ishikawa, H.; Tsumuraya, Y.; Hashimoto, Y.; Dohmae, N.; Takio, K. *J. Biochem.* **1995**, *118*, 1014.
16. Nonaka, T.; Hashimoto, Y.; Takio, K. *J. Biochem.* **1998**, *124*, 157.
17. Hori, T.; Kumasaka, T.; Yamamoto, M.; Nonaka, T.; Tanaka, N.; Hashimoto, Y.; Ueki, T.; Takio, K. *Acta Crystallogr., Sect. D* **2001**, *57*, 361.
18. Dong, S. Y.; Ma, H. M.; Duan, X. J.; Chen, X. Q.; Li, J. *J. Proteome Res.* **2005**, *4*, 161.
19. Gilmore, J. M.; Scheck, R. A.; Esser-Kahn, A. P.; Joshi, N. S.; Francis, M. B. *Angew. Chem., Int. Ed.* **2006**, *45*, 5307.
20. Sasaki, T.; Kodama, K.; Suzuki, H.; Fukuzawa, S.; Tachibana, K. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4550.
21. We first tried the isolation of N-terminal peptide through condensation with DNPH, but the modified N-terminal peptide was not obtained. Therefore, we changed DNPH for NBHA.
22. Matsuo, E.; Toda, C.; Watanabe, M.; Ojima, N.; Izumi, S.; Tanaka, K.; Tsunasawa, S.; Nishimura, O. *Proteomics* **2006**, *6*, 2042.
23. MALDI-MS spectra and tandem mass spectra (PSD mode) were obtained on an AXIMA-TOF^2 mass spectrometer (Shimadzu, Kyoto, Japan; Kratos, Manchester, UK). All analyses were performed in positive-ion reflectron mode.
24. Wachter, E.; Machleidt, W.; Hofner, H.; Otto, J. *FEBS Lett.* **1973**, *35*, 97.
25. Matrix solution was prepared by dissolving each CHCA and 3H4NBA in 0.1% TFA solution containing 50% acetonitrile at a concentration of 10 mg/ml and combining equal volumes of each solution. In addition, we used methanediphosphonic acid (MDPNA), which was reported to be effective for eliminating alkali metal adduct, as a matrix additive (Kuyama, H.; Sonomura, K.; Nishimura, O. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 1109). Hence, sample solution, matrix solution, and MDPNA solution were mixed on a MALDI target plate and air-dried (Fig. 1b and 1d).
26. Coussot, G.; Hawke, D. H.; Mularz, A.; Koomen, J. M.; Kobayashi, R. *Anal. Biochem.* **2007**, *361*, 302.
27. Scheck, R. A.; Dedeo, M. T.; Iavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2008**, *130*, 11762.